UC Merced

UC Merced Previously Published Works

Title

Evolution of TNF-induced apoptosis reveals 550 My of functional conservation.

Permalink

https://escholarship.org/uc/item/7jw4f056

Journal

Proceedings of the National Academy of Sciences of the United States of America, 111(26)

ISSN

0027-8424

Authors

Quistad, Steven D
Stotland, Aleksandr
Barott, Katie L
et al.

Publication Date

2014-07-01

DOI

10.1073/pnas.1405912111

Peer reviewed

eScholarship.org Powered by the California Digital Library
University of California

Evolution of TNF-induced apoptosis reveals 550 My of functional conservation

Steven D. Quistad[a,1], Aleksandr Stotland[a,b], Katie L. Barott[a,c], Cameron A. Smurthwaite[a], Brett Jameson Hilton[a], Juris A. Grasis[a], Roland Wolkowicz[a], and Forest L. Rohwer[a]

[a]Department of Biology, San Diego State University, San Diego, CA 92182; [b]The Cedars-Sinai Heart Institute, Los Angeles, CA 90048; and [c]Marine Biology Research Division, Scripps Institution of Oceanography, University of California, San Diego, La Jolla, CA 92093

Edited by Max D. Cooper, Emory University, Atlanta, GA, and approved May 8, 2014 (received for review March 31, 2014)

The Precambrian explosion led to the rapid appearance of most major animal phyla alive today. It has been argued that the complexity of life has steadily increased since that event. Here we challenge this hypothesis through the characterization of apoptosis in reef-building corals, representatives of some of the earliest animals. Bioinformatic analysis reveals that all of the major components of the death receptor pathway are present in coral with high-predicted structural conservation with *Homo sapiens*. The TNF receptor-ligand superfamilies (TNFRSF/TNFSF) are central mediators of the death receptor pathway, and the predicted proteome of *Acropora digitifera* contains more putative coral TNFRSF members than any organism described thus far, including humans. This high abundance of TNFRSF members, as well as the predicted structural conservation of other death receptor signaling proteins, led us to wonder what would happen if corals were exposed to a member of the human TNFSF (HuTNFα). HuTNFα was found to bind directly to coral cells, increase caspase activity, cause apoptotic blebbing and cell death, and finally induce coral bleaching. Next, immortalized human T cells (Jurkats) expressing a functional death receptor pathway (WT) and a corresponding Fas-associated death domain protein (FADD) KO cell line were exposed to a coral TNFSF member (AdTNF1) identified and purified here. AdTNF1 treatment resulted in significantly higher cell death ($P < 0.0001$) in WT Jurkats compared with the corresponding FADD KO, demonstrating that coral AdTNF1 activates the *H. sapiens* death receptor pathway. Taken together, these data show remarkable conservation of the TNF-induced apoptotic response representing 550 My of functional conservation.

evolution immunity | cytokines | Cnidarians | climate change | invertebrate immunity

Discoveries from model organisms have significantly influenced the field of human immunology. For example, the original concept of self vs. nonself recognition was discovered from observations in echinoderms, whereas the discovery of Toll-like receptors in humans stemmed from investigations into the response of insects to pathogens. Despite the impact of these studies, the majority of our understanding of immune function remains based on data from a select few taxa, mainly Chordata, Arthropoda, and Nematoda, which represent only 3 of the 30 extant animal phyla (1). Although these models have provided valuable insight into the molecular basis of immune defense, we are overlooking a significant and potentially informative portion of metazoan biology. With the rise of the genomic revolution, an increasing number of genomes from basal phyla are revealing the evolution of immunity to be a nonlinear process, involving multiple instances of gene gain and loss (2). Therefore, the investigation of nontraditional phyla will provide a deeper understanding of the evolution of immunity, including the potential for the discovery of novel immune reactions.

The phylum Cnidaria diverged from Bilateria 550 Mya and contains more than 10,000 species that range in size from a few millimeters to more than 75 m (3). Their body plan consists of two cell layers, an endoderm and ectoderm, held together by the jelly-like mesoglea (4). Stony corals (Order Scleractinia) are colonial cnidarians and are responsible for supporting the most biologically diverse ecosystem on the planet: the coral reef. Reefs support economically important industries such as fishing and tourism and provide coastal protection to hundreds of millions of people worldwide. Recent global surveys have indicated that 19% of coral reefs have been destroyed, 15% are under imminent risk of collapse, and a further 20% are under long-term threat of collapse (5). Anthropogenic impacts such as overfishing and nutrient runoff have been implicated in increased coral death and bleaching (6). However, although many of the environmental factors leading to coral mortality are well established, the biological mechanisms behind coral death remain poorly understood (7, 8).

One common route of coral death on reefs around the world occurs through a process called coral bleaching. During bleaching, the coral's intracellular symbiotic zooxanthellae are expelled from the host (9). Programmed cell death or apoptosis has been observed during the bleaching process; however the components of the apoptotic pathway have yet to be fully identified and functionally tested (10). In humans, apoptosis can be activated through either intrinsic or extrinsic pathways. The intrinsic pathway is initiated by cell stress, whereas the extrinsic pathway is initiated by death ligand/death receptor interactions. Both pathways converge on the members of the B-cell lymphoma family members (Bcl-2) family, which can ultimately lead to caspase activation and the release of signaling molecules such as cytokines to neighboring cells. This process directly links the

Significance

The TNF receptor-ligand superfamily is a central mediator of apoptosis or programmed cell death. Here we show that TNF-induced apoptosis has been functionally maintained for more than half a billion years of evolution. In response to human TNFα, coral cells underwent the classical stages of apoptosis including cellular blebbing, caspase activation, and eventual cell death. Next, the reciprocal experiment showed that coral TNF kills human cells through direct interaction with the death receptor pathway. In addition, corals were found to possess more putative TNF receptors than any organism previously described, including humans. This work provides important insight into the general evolution of apoptosis and demonstrates remarkable conservation of the TNF apoptotic response.

Author contributions: S.D.Q., A.S., R.W., and F.L.R. designed research; S.D.Q., A.S., K.L.B., C.A.S., and B.J.H. performed research; S.D.Q., A.S., C.A.S., B.J.H., and R.W. contributed new reagents/analytic tools; S.D.Q., A.S., K.L.B., C.A.S., B.J.H., J.A.G., R.W., and F.L.R. analyzed data; and S.D.Q. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

[1]To whom correspondence should be addressed. E-mail: steven.quistad@gmail.com.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1405912111/-/DCSupplemental.

apoptotic pathway with the innate immune system (11). In corals, apoptosis has been observed in response to hyperthermic oxidative stress, disease, and as a postphagocytic removal mechanism of zooxanthellae during the onset of symbiosis (10, 12). The recent *Acropora digitfera* genome suggests that coral possess homologs to the human intrinsic and extrinsic apoptotic pathways (13). Although the activation of the intrinsic apoptotic pathway in corals has been observed in response to environmental stress, the extrinsic pathway has yet to be investigated in this system. Specifically, no work to date has focused on the upstream receptor-ligand families involved with apoptotic signaling and activation.

The TNF receptor-ligand superfamily is a central mediator of the extrinsic apoptotic pathway. It is known to be involved in a variety of chronic human diseases such as multiple sclerosis, rheumatoid arthritis, and type 2 diabetes (14). The TNF ligand superfamily (TNFSF) is characterized by a ligand trimerization interface and TNF receptor binding domain. Members of the TNF receptor superfamily (TNFRSF) are defined by the presence of cysteine-rich domains (CRDs), which are important for receptor oligomerization (15). Crystal structure characterization of TNFRSF–TNFSF interactions have also revealed 50s and 90s loop structures that are important for ligand binding and specificity, respectively (15). On ligand binding, the TNFR-1 recruits TRADD, RIP1, and TRAF2, creating complex I, which dissociates from the receptor. Complex I can then activate either the NF-κB transcription factor (among others), leading to cell survival, or bind to Fas-associated death domain protein (FADD), resulting in caspase recruitment and apoptosis (16, 17).

Phylogenetic analysis indicates a deep evolutionary origin of the TNFSF and TNFRSF that precedes the divergence of vertebrates and invertebrates. The most ancient and well-defined invertebrate TNF ligand-receptor system that has been described to date is that of the fruit fly *Drosophila melangastor* (18). *D. melangastor* posseses just one member of both the TNFRSF/TNFSF, in contrast to humans who have 18 and 29, respectively (19). This difference has led to the widely accepted hypothesis that the TNF ligand-receptor superfamily expanded after the divergence of invertebrates and vertebrates (20, 21).

In this paper, we describe the annotation of 40 members of the TNFRSF and 13 members of the TNFSF in the reef building coral *A. digitifera,* suggesting that key parts of the TNF receptor-ligand superfamily have been lost in *D. melangastor* but maintained in coral (22). Comparison of these coral TNFSF/TNFRSF members to those of *Homo sapiens* reveals high genetic and predicted structural conservation. Exposure of coral to human TNFα (HuTNFα) results in apoptotic cellular blebbing, caspase activation, cell death, and finally coral bleaching. Further, we show that exposure of human T-cell lymphocytes to a coral TNFSF member identified and purified here (AdTNF1) directly activates the death receptor pathway in humans. Taken together, these data demonstrate functional conservation of TNF-induced apoptosis across 550 My of evolution. This work also identifies, to our knowledge, the first ligand-receptor signaling pathway to be directly involved in the activation of bleaching and apoptosis in coral. Because coral bleaching events are expected to increase in frequency with future climate change, improving our understanding of the molecular mechanisms involved is prudent for reef conservation and our understanding of the general evolution of apoptosis (23).

Results

Bioinformatic Analysis of the *A. digitifera* Apoptotic Repertoire Reveals High-Predicted Conservation. To elucidate the complexity of the coral apoptotic repertoire, we used the recently published genome of *A. digitifiera* (13). Thirty-one putative TNF receptor-associated factors (TRAFs) with an average length of 458 amino acids were found to have high conservation with *H. sapiens* TRAF1 within the TRAF family domain. (Fig. S1*A* and Table S1). Twenty putative caspases with an average length of 533 amino acids were found to have high conservation within the α/β fold regions of *H. sapiens* caspase-3, including residues located within the caspase-3 active site (Fig. S1*B* and Table S1). Thirteen members of the TNFSF with an average length of 228 amino acids were found to have high conservation with *H. sapiens* TNFα (HuTNFα) with the TNF ligand superfamily domain (Fig. 1*A* and Table S2). Forty putative members of the TNFRSF with an average length of 508 amino acids were also identified within the genome of *A. digitifera* (Table S3). All AdTNFRs contained a minimum of one 50s TNF-binding loop (ligand binding specificity) and one 90s binding loop (receptor oligimerization). The total number of CRDs ranged from zero to four. Eleven of the putative TNFRSF's proteins contained death domains (AdTNFR1–AdTNFR11), whereas five contained Ig domains (AdTNFR18–AdTNFR22). Structural threading of AdTNF1/AdTNFR1 with two members of the human TNFSF/TNFRSF, CD40L and CD40, respectively, suggests high-predicted homology (Fig. 1 *B* and *C*). Compared with previously published work on members of the TNFRSF, corals contain the most diverse TNFRSF repertoire of any organism described to date, including humans (Table S4). *A. digitifera* also possess other canonical apoptotic proteins including Bcl-2 members (8), inhibitors of apoptosis (4), APAF-1, FADD, and cytochrome *c* (Fig. 1*D*). Fig. S2 shows the phylogenetic relationships between AdTNFs, AdTNFRs, AdCasps, and AdTRAFs and *H. sapiens* homologs.

HuTNFα Causes Apoptosis in *Acropora yongeii*. To investigate whether HuTNFα affects coral protein expression, we used Human Explorer Antibody Arrays (Full Moon Biosystems) and found that HuTNFα led to dynamic changes of unknown coral proteins bound to human antibodies to Bcl-$X_{L,}$ Fas, CD40, and multiple CD-receptors (Figs. S3 and S4). To characterize the cellular response of coral to HuTNFα, we first performed immunohistochemistry to demonstrate that HuTNFα binds directly to coral cells (Fig. 2 *A* and *B*). Next we exposed a 20-μm cultured coral cell to HuTNFα under live confocal microcopy and found evidence of apoptotic blebbing within 10 min of treatment (Fig. 2*C*). Quantification of a second 7-μm cell type extracted from adult coral tissue revealed an increase in the number of visible apoptotic cells after 90 min of HuTNFα exposure (Fig. 2*D*). HuTNFα caused a shift in the percentage of apoptotic cells from ∼15% in the untreated coral cells to ∼75% in the HuTNFα-treated cells (n = 200 cells counted; Fig. 2*E*). HuTNFα exposure was also found to significantly increase ($P < 0.0001$) caspase activity of extracted coral cells relative to a negative control inhibitor (Z-FA-FMK; BD Pharmigen) (Fig. 2*F*). Furthermore, 4 h of HuTNFα exposure resulted in a significant ($P < 0.001$) increase in the total number of dead coral cells (Fig. 2*G*). Taken together, these data support the hypothesis that HuTNFα causes apoptosis in the reef-building coral *A. yongeii*.

HuTNFα Causes Myosin Fragmentation and Results in an Acidic Shift in the Coral Proteome. To further examine the effect of HuTNFα on the coral proteome, protein was extracted from untreated coral and coral exposed to HuTNFα and analyzed with 2D gel electrophoresis and LC-MS. Following 30 min of HuTNFα stimulation, 92 spots were found to be significantly different ($P < 0.05$) between the untreated and HuTNFα gels (Fig. 3*A*), with an observed isoelectric shift toward more acidic proteins (Fig. 3*B*). Mass spectra data were compared with a custom-built coral protein database created from the predicted proteome (13). Four separate spots were identified as coral myosin (adi_v1.18643), which increased on HuTNFα exposure (Fig. 3*A*, spots 2, 3, 5, and 6). The predicted molecular mass of coral myosin is ∼316 kDa, whereas the four identified myosin spots ranged from 70 to 84 kDa, suggesting cleavage. In addition, the banding pattern

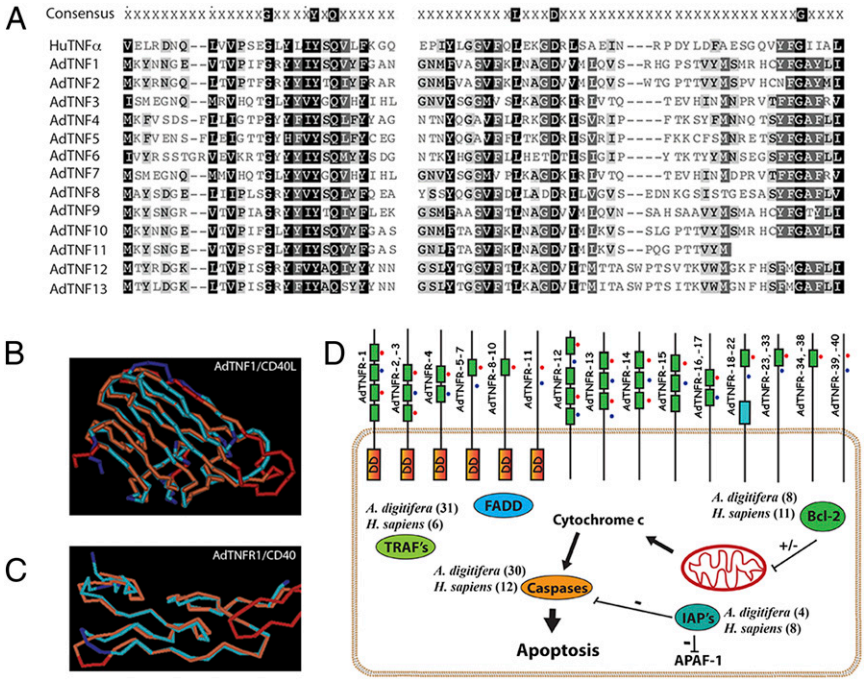

Fig. 1. Bioinformatic analysis of coral and human proteins involved with death receptor signaling. (*A*) Primary sequence alignment of putative *A. digitifera* TNF ligands with *H. sapiens* TNFα. (*B*) Predicted structural alignment of AdTNF1 (residues 25–161) and *H. sapiens* CD40L. Light blue and orange = high predicted structural homology, dark red = CD40L, and dark blue = AdTNF1 (*C*). Predicted structural alignment of AdTNFR1 (residues 19–79) with *H. sapiens* CD40. Light orange and light blue = high-predicted structural homology, dark red = CD40, and dark blue = AdTNFR1. (*D*) The putative TNFR repertoire of *A. digitifera* (*Upper*) with death domain (DD), cysteine-rich domain (green boxes), immunoglobin domain (blue box), 50s loop TNF binding site (red dot), and 90s loop TNF binding site (blue dot) indicated. Members of the death receptor signaling pathway (*Lower*) found in the *A. digitifera* genome with number of proteins within a specific protein family indicated for both *A. digitifera* and *H. sapiens* including TNF receptor associated factors (TRAFs), B-cell lymphoma family members (Bcl-2), inhibitor of apoptosis proteins (IAPs), FADD, APAF-1, and caspases.

observed in Fig. 3*C* is suggestive of myosin phosphorylation. The initial induction of apoptosis also affects calcium signaling (24), and following HuTNFα exposure, two proteins that contain an EF-hand calcium binding site and calreticulin were found to be up-regulated (Table S5), suggesting that HuTNFα affects calcium signaling in corals. Finally the Zooxanthallae-specific protein Peridinin was also up-regulated, providing initial evidence that HuTNFα affects coral-algal symbiosis (Fig. 3*C* and Table S5).

HuTNFα Causes Bleaching of *A. yongeii*. To test whether HuTNFα-induced apoptosis is also involved in coral bleaching, we used a flow cytometric approach. Coral tissue was found to expel zooxanthellae in the presence and absence of treatment with HuTNFα (Fig. S5 *A* and *B*). Untreated coral initially expelled more zooxanthellae after 1 h than HuTNFα-treated coral; however, after 7 h, the HuTNFα-treated coral expelled ∼200% more algae than the untreated coral (Fig. S5*C*). When both untreated and HuTNFα-treated corals were exposed to 100 mg/mL of ampicillin (AMP), the untreated control initially released more zooxanthallae than the HuTNFα-treated coral. However, at 10 h, the +HuTNFα/+AMP-treated coral had ∼400% more expelled algae, which increased to ∼500% by 12 h (Fig. S5*D*). From 6 to 12 h, the zooxanthellae released by the −HuTNFα/+AMP coral remained relatively constant at ∼2,000 expelled algal cells (Fig. S5*D*).

Crude AdTNF1 Causes Apoptosis in Coral and Is Involved with Bleaching. To conduct preliminary investigations into the biological effects of one of the newly described coral TNF ligands (AdTNF1), we created a construct with a Prolactin signal sequence fused to AdTNF1 ensuring its secretion into the surrounding media (PBMN.i.mChAdTNF1) (25). A diagram representing the constructs for the secretion of GFP and AdTNF1 is presented in Fig. S5*A*. As a control, a construct with GFP fused to a Prolactin signal sequence was also created (pBMN.i.mChGFP). 293T cells transfected with pBMN.i.mChGFP displayed stable expression of GFP localized to the endoplasmic reticulum/trans-Golgi, as expected of secreted proteins (Fig. S6*B*). Exposure of coral cells to 10 μL of media from 293T cells transfected with pBMN.i.mChAdGFP control resulted in ∼20% apoptotic cells, whereas cells exposed to media from the pBMN.i.mChAdTNF1 resulted in ∼80% apoptotic cells (n = 200; Fig. S5*C*). Adult coral tissue exposed to 250 μL of pBMN.i.mChAdTNF1 media resulted in a significant ($P < 0.05$) reduction in total algae expelled at 4 h posttreatment; however, there was no significant difference from the control by 6 h (Fig. S6*D*). Finally, the FLAG (26) epitope was cloned into the C terminus of AdTNF1, and evidence for direct binding of AdTNF1-FLAG to coral cells is presented in Fig. S6 *E* and *F*.

Purified Coral AdTNF1 Causes Apoptosis in Human T-Lymphocytes. To directly test whether coral AdTNF1 interacts with the human death receptor pathway, we used WT immortalized human T lymphocytes (Jurkats) and a corresponding FADD KO cell line (ATCC CRL-2572; Fig. 4*A*). AdTNF1 was further purified through His-tag nickel affinity chromatography (Fig. 4*B*; Monserate Biotechnology Group) and used for subsequent experimentation. Bioinformatic analysis demonstrates high-predicted structural conservation between AdTNF1 and FasL (27) (Fig. 4*C*). Propidium iodide staining demonstrated that FasL negatively affects WT cell viability in a dose-dependent manner, whereas FADD KO cell viability is unaffected (Fig. S7*A*). Next we exposed both WT and FADD KO cells to AdTNF1. Following 48 h of AdTNF1 treatment, WT Jurkat cells exhibited a significant ($P < 0.0001$) reduction in cell viability compared with the FADD KO cells,

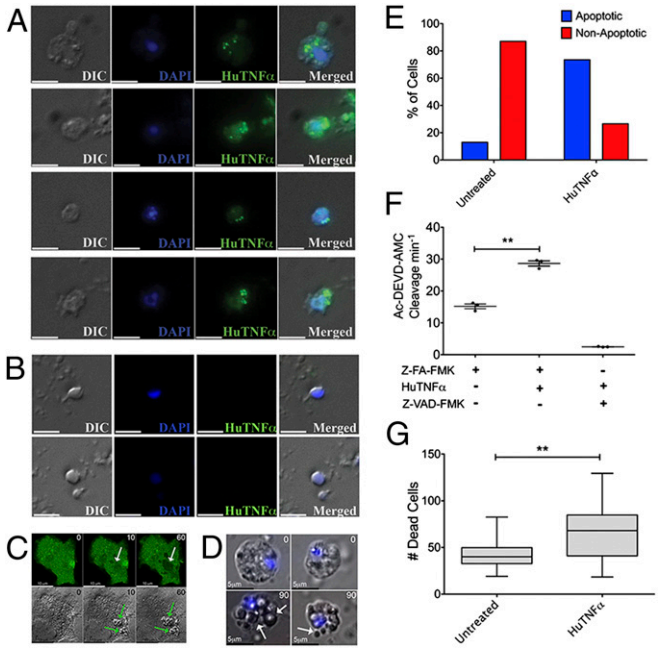

Fig. 2. Effect of HuTNFα on *A. yongeii* cell populations. (*A*) Representative coral cells incubated with HuTNFα and HuTNFα antibody, stained with DAPI. (White bar, 5 μm.) (*B*) Representative coral cells incubated with HuTNFα antibody and stained with DAPI. (White bar, 5 μm.) (*C*) Live confocal microscopy of a GFP autoflourescent coral cell exposed to HuTNFα at 0, 10, and 60 min. (White bar, 10 μm.) White/green arrow indicates regions of apoptotic body formation. (*D*) Representative images of apparently healthy coral cells (*Upper*) and apoptotic cells (*Lower*) stained with DAPI at 0 and 90 min. (Black bar, 5 μm.) White arrows indicate apoptotic bodies. (*E*) Relative percentages of apoptotic/nonapoptotic coral cell populations left untreated or incubated with HuTNFα for 90 min (n = 200 cells). (*F*) Caspase activity of coral cells stimulated with HuTNFα for 30 min with negative control inhibitors (Z-FA-FMK) and pan-caspase inhibitors (Z-VAD-FMK) indicated (**P = 0.0024; unpaired t test with ±SEM indicated). (*G*) Total number of dead cells per image (n = 32 images) under untreated conditions and HuTNFα exposure for 4 h with interquartile ranges (boxes) and whiskers (10–90 percentiles) indicated (**P = 0.0011; unpaired t test).

demonstrating that coral AdTNF1 directly interacts with the death receptor pathway in humans, increasing cell death (Fig. 4*D* and Fig. S7*B*).

Discussion

HuTNFα exposure led to increased caspase activity, cellular blebbing, and cell death, demonstrating that HuTNFα causes apoptosis in coral. Furthermore, AdTNF1 was found to directly interact with the *H. sapiens* death receptor pathway, also resulting in cell death. This suggests that apoptotic signaling through TNFRSF/TNFSF proteins was fully functional at the time of the pre-Cambrian explosion, and remarkably, the domains necessary to activate apoptosis have been maintained from corals to humans.

A recent review by Weins et al. (21) explored the origin and evolution of the TNF receptor-ligand superfamilies and concluded that their evolutionary origin could be traced back to single copy genes within arthropods. They posit that these founding genes underwent multiple duplication events following the divergence of invertebrates and vertebrates, which coincided with the development of the adaptive immune system (21). However, this study failed to take into account the recently published Cnidarian genomes of *Nematostella vectensis* and *A. digitifera* (2, 13). The existence of 40 putative coral TNF receptors (AdTNFR1–AdTNFR40) and 13 putative coral TNF ligands (AdTNF1–AdTNF13) identified here suggests a far more ancient origin of the TNF receptor-ligand superfamily that precedes arthropods. Our data demonstrate high conservation of TNFRSF/TNFSF members in corals, suggesting the last common metazoan ancestor possessed a functional TNF apoptotic pathway, which subsequently underwent gene reduction in arthropods and other invertebrates. Corals possess more putative members of the TNFRSF of any organism described thus far and possess a similar number of putative TNFSF proteins as many vertebrates (Table S5) (21). Although the function of AdTNFR1–AdTNFR40 and AdTNF1–AdTNF13 still requires elucidation, the TNF receptor-ligand superfamily has clearly undergone dynamic changes throughout the various lineages of metazoan evolution, independent of a particular phylum's structural complexity. Similar complexity has also been observed in the Cnidarian *N. vectensis* within the Nme and Wnt gene families, a complexity that has also been lost in other model ecdyzoans (28, 29). These studies, along with ours, highlight the need to take into account the genomes of a broad range of animal phyla before we can draw broad conclusions about the evolution of gene families.

Beyond the specific TNF ligand-receptor pathway, the general existence of cytokines in invertebrates has been argued to be the result of convergent evolution (30–32). For example, in *D. melangastor*, the Toll-like receptor pathway is involved in the response to microbial infection. On immune stimulation, protease cascades lead to the activation of the cytokine Spatzle (33). In humans, the related pathway involves the Toll-like receptor (IL1-R1) and its respective ligand (IL-1) (34). Although both the *D. melangastor* Toll and human IL-1 pathways converge on the activation of NF-κB transcription factor homologs, IL-1 and Spatzle do not show any significant similarity at the amino acid level. Furthermore, the completed genome of *D. melangastor* failed to reveal any proteins homologous to human IL-1. From these data, it was concluded that invertebrates lack any

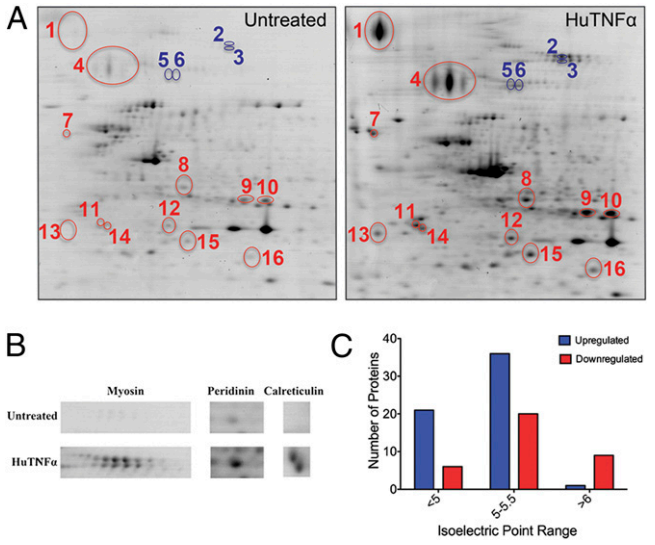

Fig. 3. Proteomic analysis of coral exposed to HuTNFα using 2D gel electrophoresis. (*A*) 2D gel electrophoresis of extracted protein from untreated coral (*Left*) and human TNFα-stimulated coral (*Right*) for 30 min. Numbered circles indicate proteins that were identified through MS, whereas blue circles indicate fragments of myosin. (*B*) Representative proteins that were up-regulated in response to HuTNFα including myosin, the zooxanthallae-specific protein peridinin, and calreticulin. (*C*) Ninety-two proteins that were significantly different between the untreated and human TNFα-treated gels grouped by their respective isoelectric points. Red bar indicate the number of proteins in a specific p*I* range that were down-regulated in response to HuTNFα stimulation. Blue bars represent the number of proteins in a specific p*I* range that were up-regulated in response to HuTNFα stimulation.

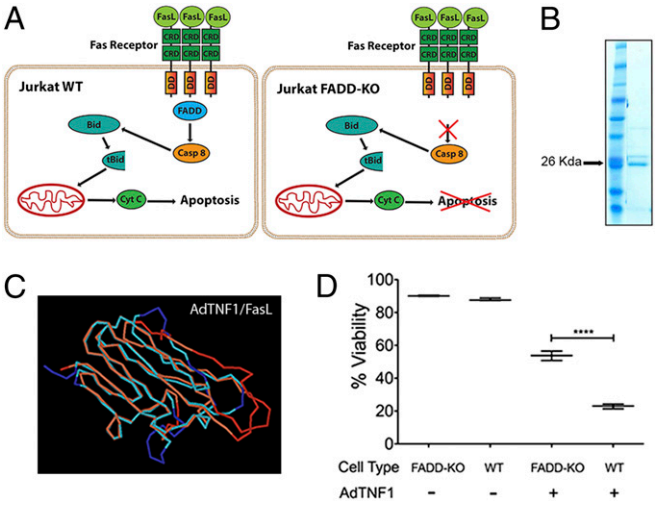

Fig. 4. Effect of AdTNF1 on human T-lymphocyte cellular viability. (*A*) T-cell lymphoma cell lines (Jurkat) used for experimentation. WT cells are sensitive to Fas-induced apoptosis, whereas FADD KO are resistant. (*B*) Production and isolation of His-tagged AdTNF1 through Nickel-affinity chromatography with correct size of His-AdTNF1 indicated. (*C*) Predicted structural alignment of FasL and AdTNF1. Light orange and light blue = high-predicted structural homology, dark red = FasL, and dark blue = AdTNFR1. (*D*) The effect of AdTNF1 on cellular viability of WT and FADD KO Jurkat cell lines (****$P <$ 0.0001; unpaired *t* test).

homologous pathway to vertebrate IL-1/IL-1-R signaling. Beyond the specific IL-1 pathway, multiple vertebrate and invertebrate cytokines have been shown to exhibit similar biological functions, yet they lack any genetic homology (30). It was therefore postulated that the similar biologically activities of these cytokines is a result of convergent evolution. However, the vast majority of data supporting this hypothesis are taken from the model systems of *D. melangastor* and *Caenorhabditis elegans*, which as discussed above have lost complexity in multiple gene networks. The high amino acid conservation between coral TNFRSF members and HuTNFα, as well as the activation of apoptosis in coral using a human cytokine, support the hypothesis of a divergent evolution of the TNF receptor-ligand superfamily. Future work should focus on the cytokine repertoire of other phyla to develop a comprehensive hypothesis of metazoan cytokine evolution.

The canonical apoptotic cascade is executed by a group of cysteine-dependent aspartate-directed proteases known as caspases, which, on activation by the adaptor protein FADD, cleave various cellular substrates, leading to apoptotic body formation and eventual cell death. Within FADD, two essential domains designated the death domain (DD) and death effector domain (DED) are required for apoptotic transduction (35). A putative FADD protein containing the DD and DED domains has been identified in both *Hydra* and the *A. digitifera* proteome (aug_v2a.04795) (36, 37). On activation of apoptosis, the phosphorylation and cleavage of the myosin-light chain are critical for the subsequent morphological changes involved with cellular blebbing (38). Fragments of coral myosin were found to significantly increase on HuTNFα stimulation, and the associated banding pattern in response to HuTNFα is suggestive of a phosphorylation event (Fig. 3*C*). Furthermore, the acidic shift of the 92 proteins could also be the result of a larger phosphorylation cascade (Fig. 3*B*) (39). Interestingly, one of the most highly up-regulated proteins in the HuTNFα-stimulated gel contained a Zona-Pellucida (ZP) domain, which has traditionally been studied within the context of fertilization (40). Although ZP domain proteins have not been well studied within the context of TNF signaling in humans, preexposure of sperm to HuTNFα impairs sperm binding (41). The role of ZP domain proteins in the coral TNF signaling cascade should be a focus of future studies.

Although previous studies have demonstrated apoptotic coral cells in whole coral tissue, to the authors' knowledge, Fig. 2 reveals the first images of an isolated coral cell undergoing cytokine-induced apoptosis (42). We hypothesize that HuTNFα binds to one of the AdTNF receptors containing a death domain (AdTNF1–AdTNF6), initiating the apoptotic cascade and caspase activation (Fig. 2*F*). A biochemical model of bleaching has been proposed in which reactive oxygen species (ROS) production by the algal symbionts compromises the structural integrity of the mitochondrial membrane, stimulating the release of apoptotic factors and caspase activation (43). With the identification of a diverse repertoire of 40 putative TNF receptors and 13 putative TNF ligands described here, as well as the potential involvement of the mitochondria on HuTNFα exposure (Fig. S3*C*), we propose supplementing this model with further investigation into the specific members of the coral TNFRSF/TNFSF and their involvement in bleaching and apoptotic processes.

Previous investigations into the mechanism of coral bleaching have largely relied on thermal stress to induce zooxanthellae expulsion. Although environmentally relevant, the application of thermal stress causes dynamic changes to the coral holobiont making a determination of the specific signaling pathways directly involved in apoptosis and bleaching challenging if not impossible (44). In this study, we induced both of these cellular processes through the application of a single protein to adult coral tissue and individual coral cells. Although previous work has investigated the downstream effectors of apoptosis such as caspases (43) and Bcl-2 family members (42), this is the first examination, to our knowledge, of the upstream ligands and receptors involved with initiating apoptosis in coral. Recently published transcriptomic studies of corals exposed to various environmental stressors have implicated members of the TNFSF/TNFRSF, as well as downstream proteins involved with apoptosis, supporting the ecological relevance of the TNF pathway in coral (45–49).

This study reveals an ancient origin of the TNF receptor-ligand superfamily. The activation of apoptosis in coral using a human TNF ligand (Fig. 2) in conjunction with the induction of apoptosis in humans using coral AdTNF1 (Fig. 4) demonstrates remarkable evolutionary conservation that has been functionally maintained for 550 My. Although we demonstrate that AdTNF1 specifically interacts with the death receptor pathway in humans, the mechanism remains unknown. Furthermore, the existence of 12 additional coral TNF ligands (AdTNF2–AdTNF13) and the interactions of those TNF ligands with human cell physiology create exciting possibilities for future research.

Materials and Methods

Members of the coral TNFSF/TNFRSF were bioinformatically extracted from the *A. digitifera* proteome (13) and analyzed using the DAS transmembrane prediction server (50), PRO-SITE database (51), and the Conserved Domain Database (52). Coral cell culture methods were adapted from Helman et al. (53) and Reyes-Bermudez and Miller (54) and utilized for immunohistochemistry, caspase activity, and live-dead cell assays. AdTNF1 was purified through His-tag nickel affinity chromatography (Monserate Biotechnology Group) and used for subsequent experimentation. Jurkat WT and Jurkat FADD KO cells (ATCC CRL-2572) were exposed to AdTNF1, stained with propidium iodide, and sorted through flow cytometry. See *SI Materials and Methods* for further details.

ACKNOWLEDGMENTS. We thank Leo Su and the Monserate Biotechnology Group for the purification of AdTNF1, Fernando Nosratpour and Vince Levesque at the Birch Aquarium for their generous donation of *A. yongeii*, Phillip Cash (Aberdeen Proteomics) for 2D-Gel analysis, Rob Edwards for array suggestions, Nikki Traylor-Knowles for helpful comments with the manuscript, and the San Diego State University FACS facility. This work was funded by a National Science Foundation Graduate Research fellowship (to S.D.Q.), National Institutes of Health Grant 1 R21 AI094534-01 (to F.L.R.), and the Canadian Institute For Advanced Research Integrated Microbial Diversity Fellowship (IMB-ROHW-141679) (to F.L.R.).

1. Litman GW, Cooper MD (2007) Why study the evolution of immunity? *Nat Immunol* 8(6):547–548.
2. Putnam NH, et al. (2007) Sea anemone genome reveals ancestral eumetazoan gene repertoire and genomic organization. *Science* 317(5834):86–94.
3. Daly M, et al. (2007) The phylum Cnidaria: A review of phylogenetic patterns and diversity 300 years after Linnaeus. *Zootaxa* 1668:127–182.
4. Augustin R, Bosch TCG (2010) Cnidarian immunity: A tale of two barriers. *Adv Exp Med Biol* 708:1–16.
5. Bourne DG, et al. (2009) Microbial disease and the coral holobiont. *Trends Microbiol* 17(12):554–562.
6. Brown BE (1997) Coral bleaching: Causes and consequences. *Coral Reefs* 16-(SuppI):S129–S138.
7. Rosenberg E, Kushmaro A, Kramarsky-Winter E, Banin E, Yossi L (2009) The role of microorganisms in coral bleaching. *ISME J* 3(2):139–146.
8. Lesser M (2011) Coral bleaching: Causes and mechanisms. *Coral Reefs: An Ecosystem in Transition*, eds Dubinsky Z, Stambler N (Springer, Houten, The Netherlands), pp 405–419.
9. Davy SK, Allemand D, Weis VM (2012) Cell biology of cnidarian-dinoflagellate symbiosis. *Microbiol Mol Biol Rev* 76(2):229–261.
10. Dunn SR, Schnitzler CE, Weis VM (2007) Apoptosis and autophagy as mechanisms of dinoflagellate symbiont release during cnidarian bleaching: Every which way you lose. *Proc Biol Sci* 274(1629):3079–3085.
11. Zhou F, Yang Y, Xing D (2011) Bcl-2 and Bcl-xL play important roles in the crosstalk between autophagy and apoptosis. *FEBS J* 278(3):403–413.
12. Weis VM (2008) Cellular mechanisms of Cnidarian bleaching: Stress causes the collapse of symbiosis. *J Exp Biol* 211(Pt 19):3059–3066.
13. Shinzato C, et al. (2011) Using the Acropora digitifera genome to understand coral responses to environmental change. *Nature* 476(7360):320–323.
14. Bahia MS, Silakari O (2010) Tumor necrosis factor alpha converting enzyme: An encouraging target for various inflammatory disorders. *Chem Biol Drug Des* 75(5): 415–443.
15. Hymowitz SG, et al. (1999) Triggering cell death: The crystal structure of Apo2L/TRAIL in a complex with death receptor 5. *Mol Cell* 4(4):563–571.
16. Lin Y, Devin A, Rodriguez Y, Liu ZG (1999) Cleavage of the death domain kinase RIP by caspase-8 prompts TNF-induced apoptosis. *Genes Dev* 13(19):2514–2526.
17. Micheau O, Tschopp J (2003) Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. *Cell* 114(2):181–190.
18. Kauppila S, et al. (2003) Eiger and its receptor, Wengen, comprise a TNF-like system in Drosophila. *Oncogene* 22(31):4860–4867.
19. Moreno E, Yan M, Basler K (2002) Evolution of TNF signaling mechanisms: JNK-dependent apoptosis triggered by Eiger, the Drosophila homolog of the TNF superfamily. *Curr Biol* 12(14):1263–1268.
20. Collette Y, Gilles A, Pontarotti P, Olive D (2003) A co-evolution perspective of the TNFSF and TNFRSF families in the immune system. *Trends Immunol* 24(7):387–394.
21. Wiens GD, Glenney GW (2011) Origin and evolution of TNF and TNF receptor superfamilies. *Dev Comp Immunol* 35(12):1324–1335.
22. Robertson AJ, et al. (2006) The genomic underpinnings of apoptosis in Strongylocentrotus purpuratus. *Dev Biol* 300(1):321–334.
23. Pandolfi JM, et al. (2003) Global trajectories of the long-term decline of coral reef ecosystems. *Science* 301(5635):955–958.
24. Hajnóczky G, Davies E, Madesh M (2003) Calcium signaling and apoptosis. *Biochem Biophys Res Commun* 304(3):445–454.
25. Freeman ME, Kanyicska B, Lerant A, Nagy G (2000) Prolactin: Structure, function, and regulation of secretion. *Physiol Rev* 80(4):1523–1631.
26. Einhauer A, Jungbauer A (2001) The FLAGTM peptide, a versatile fusion tag for the purification of recombinant proteins. *J Biochem Bioph Meth* 49(1-3):455–465.
27. Sen CK, Sashwati R, Packer L (1999) Fas mediated apoptosis of human Jurkat T-cells: Intracellular events and potentiation by redox-active alpha-lipoic acid. *Cell Death Differ* 6(5):481–491.
28. Kusserow A, et al. (2005) Unexpected complexity of the Wnt gene family in a sea anemone. *Nature* 433(7022):156–160.
29. Desvignes T, Pontarotti P, Bobe J (2010) Nme gene family evolutionary history reveals pre-metazoan origins and high conservation between humans and the sea anemone, Nematostella vectensis. *PLoS ONE* 5(11):e15506.
30. Beschin A, et al. (1999) Convergent evolution of cytokines. *Nature* 400(6745):627–628.
31. Beschin A, Bilej M, Torreele E, De Baetselier P (2001) On the existence of cytokines in invertebrates. *Cell Mol Life Sci* 58(5-6):801–814.
32. Scapigliati G, Buonocore F, Mazzini M (2006) Biological activity of cytokines: An evolutionary perspective. *Curr Pharm Des* 12(24):3071–3081.
33. Valanne S, Wang JH, Rämet M (2011) The Drosophila Toll signaling pathway. *J Immunol* 186(2):649–656.
34. Dinarello CA (1996) Biologic basis for interleukin-1 in disease. *Blood* 87(6):2095–2147.
35. Tourneur L, Chiocchia G (2010) FADD: A regulator of life and death. *Trends Immunol* 31(7):260–269.
36. Lasi M, David CN, Böttger A (2010) Apoptosis in pre-Bilaterians: Hydra as a model. *Apoptosis* 15(3):269–278.
37. Koyanagi R, et al. (2013) MarinegenomicsDB: An integrated genome viewer for community-based annotation of genomes. *Zoolog Sci* 30(10):797–800.
38. Petrache I, et al. (2003) Caspase-dependent cleavage of myosin light chain kinase (MLCK) is involved in TNF-alpha-mediated bovine pulmonary endothelial cell apoptosis. *FASEB J* 17(3):407–416.
39. Anderson JC, Peck SC (2008) A simple and rapid technique for detecting protein phosphorylation using one-dimensional isoelectric focusing gels and immunoblot analysis. *Plant J* 55(5):881–885.
40. Jovine L, Darie CC, Litscher ES, Wassarman PM (2005) Zona pellucida domain proteins. *Annu Rev Biochem* 74:83–114.
41. Faber BM, Chegini N, Mahony MC, Coddington CC III (2001) Macrophage secretory products and sperm zona pellucida binding. *Obstet Gynecol* 98(4):668–673.
42. Pernice M, et al. (2011) Regulation of apoptotic mediators reveals dynamic responses to thermal stress in the reef building coral Acropora millepora. *PLoS ONE* 6(1):e16095.
43. Tchernov D, et al. (2011) Apoptosis and the selective survival of host animals following thermal bleaching in zooxanthellate corals. *Proc Natl Acad Sci USA* 108(24): 9905–9909.
44. Portune KJ, Voolstra CR, Medina M, Szmant AM (2010) Development and heat stress-induced transcriptomic changes during embryogenesis of the scleractinian coral Acropora palmata. *Mar Genomics* 3(1):51–62.
45. DeSalvo MK, et al. (2010) Coral host transcriptomic states are correlated with Symbiodinium genotypes. *Mol Ecol* 19(6):1174–1186.
46. Reyes-Bermudez A, et al. (2009) Gene expression microarray analysis encompassing metamorphosis and the onset of calcification in the scleractinian coral Montastraea faveolata. *Mar Genomics* 2(3-4):149–159.
47. Ganot P, et al. (2011) Adaptations to endosymbiosis in a cnidarian-dinoflagellate association: Differential gene expression and specific gene duplications. *PLoS Genet* 7(7):e1002187.
48. Aranda M, et al. (2011) Differential sensitivity of coral larvae to natural levels of ultraviolet radiation during the onset of larval competence. *Mol Ecol* 20(14):2955–2972.
49. Barshis DJ, et al. (2013) Genomic basis for coral resilience to climate change. *Proc Natl Acad Sci USA* 110(4):1387–1392.
50. Cserzö M, Wallin E, Simon I, von Heijne G, Elofsson A (1997) Prediction of transmembrane alpha-helices in prokaryotic membrane proteins: The dense alignment surface method. *Protein Eng* 10(6):673–676.
51. Hulo N, et al. (2006) The PROSITE database. *Nucleic Acids Res* 34(Database issue): D227–D230.
52. Derbyshire MK, Lanczycki CJ, Bryant SH, Marchler-Bauer A (2012) Annotation of functional sites with the Conserved Domain Database. *Database (Oxford)* 2012: bar058.
53. Helman Y, et al. (2008) Extracellular matrix production and calcium carbonate precipitation by coral cells in vitro. *Proc Natl Acad Sci USA* 105(1):54–58.
54. Reyes-Bermudez A, Miller DJ (2009) In vitro culture of cells derived from larvae of the staghorn coral Acropora millepora. *Coral Reefs* 28(4):859–864.